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PATENT APPLICATION

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In re application of

Hisashi NARIMATSU *et al.* Atty. Ref.: Q83405

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Filed: September 3, 2004 Examiner: Chowdhury, Iqbal Hossain

For: SULFOTRANSFERASE, PEPTIDE THEREOF AND DNA ENCODING THE SAME

DECLARATION

Commissioner for Patents
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Sir/Madam:

I, Eiichi Kobayashi, do declare and state that:

I graduated from the University of Tokyo, Faculty of Agriculture, Department in Agricultural Chemistry, having received a Master's Degree of Agriculture in March, 1992.

I understand the Japanese and English languages.

I understand the Japanese and English languages. Attachment is an accurate English translation made by me of a Japanese patent application No. 2002-057527 filed March 4, 2002.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date : November 15, 2006 Name : Kobayashi

Eiichi Kobayashi

JAPAN PATENT OFFICE

This is to certify that the annexed is a true copy of
the following application as filed with this Office.

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Specification

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J200200400

(Title of the invention)

Sulfotransferase, peptide thereof and DNA encoding the same

(Scope of the claims)

(Claim 1) A polypeptide which comprises the amino acid sequence represented by SEQ ID NO:2, or a polypeptide of a sulfotransferase which comprises an amino acid sequence having substitution, deletion, insertion or transposition of at least one amino acid in the amino acid sequence and has activity of transferring a sulfate group from a sulfate group donor to a glycosaminoglycan which is a sulfate group acceptor.

(Claim 2) The polypeptide according to claim 1, which consists of the amino acid sequence represented by SEQ ID NO:2.

(Claim 3) The polypeptide according to claim 1, which consists of amino acid numbers 37 to 346 in the amino acid sequence represented by SEQ ID NO:2.

(Claim 4) The polypeptide according to any one of claims 1 to 3, wherein the glycosaminoglycan is heparin or heparan sulfate.

(Claim 5) A sulfotransferase which comprises the polypeptide according to any one of claims 1 to 4 and has activity of transferring a sulfate group from a sulfate group donor to a glycosaminoglycan which is a sulfate group acceptor.

(Claim 6) A nucleic acid which encodes the polypeptide according to any one of claims 1 to 4 or the sulfotransferase according to claim 5.

(Claim 7) A nucleic acid which consists of the nucleotide sequence represented by SEQ ID NO:1.

(Claim 8) A nucleic acid which hybridizes with the nucleic acid according to claim 6 or 7 or a nucleic acid consisting of a nucleotide sequence complementary to the nucleotide sequence under stringent conditions.

(Claim 9) An expression vector which comprises the nucleic acid according to any one of claims 6 to 8.

(Claim 10) A recombinant which comprises the expression vector according to claim 9.

(Claim 11) A recombinant which comprises a host cell which is a eucaryotic cell into which the expression vector according to claim 9 is introduced.

(Claim 12) A process for producing a polypeptide or a sulfotransferase, which comprises culturing the recombinant according to claim 10 or 11, and recovering the polypeptide according to any one of claims 1 to 4 or the sulfotransferase according to claim 5 from the obtained culture.

(Detailed description of the invention)

(0001)

(Technical filed to which the invention belongs)

The present invention relates to a glycosaminoglycan sulfotransferase, a peptide thereof, and a nucleic acid comprising a nucleotide sequence encoding the same. More specifically, the present invention relates to a polypeptide having activity of transferring a sulfate group from a sulfate group donor to a heparin or heparan sulfate which is a sulfate group acceptor, an enzyme comprising the polypeptide, a nucleic acid comprising a nucleotide sequence encoding the polypeptide, a vector or a recombinant comprising the nucleic acid, and a process for producing the polypeptide from the recombinant.

(0002)

Heparan sulfate is a kind of glycosaminoglycan having a repeating structure of a disaccharide (4GlcAβ1/IdoAα1→4GlcNAcα1) of a hexuronic acid (HexA) residue (D-glucuronic acid (hereinafter sometimes simply referred to "glucuronic acid" or "GlcA") residue or L-iduronic acid (hereinafter sometimes simply referred to as "iduronic acid" or "IdoA") residue) and an N-acetyl-D-glucosamine (hereinafter sometimes simply referred to as "GlcNAc") residue as the basal skeleton (this basal skeleton may be also referred to as "heparin skeleton" hereinafter), wherein one or more of the 2-position hydroxyl group of its HexA residue and the 2-position amino group, the 3-position hydroxyl group and the 6-position hydroxyl group of its GlcN residue are sulfated.

(0003)

Since the gene encoding a glycosaminoglycan sulfotransferase has been cloned, it is considered that information on the substrate specificity of the enzyme for glycosaminoglycan as the sulfate group acceptor can be obtained by preparing the enzyme in a large amount, which will provide a useful approach in studying relationship between the structure and the function of glycosaminoglycan. It is known that there are many sulfation processes in the synthesis of glycosaminoglycan, particularly in the synthesis of heparin/heparan sulfate (*Glycotechnology*, (5), 57 (1994), published by Kodansha Scientific), and it is considered that various types of glycosaminoglycan sulfotransferases are concerned in this sulfation. Regarding the glycosaminoglycan sulfotransferase which transfers a sulfate group to heparin/heparan sulfate, heparan sulfate N-deacetyl/N-sulfotransferase (hereinafter sometimes referred to as "NDST"), heparan sulfate 2-O-sulfotransferase (hereinafter sometimes referred to as "HS2ST"), heparan sulfate 3-O-sulfotransferase (hereinafter sometimes referred to as "HS3OST"), heparan sulfate 6-O-sulfotransferase (hereinafter sometimes referred to as "HS6ST")

and the like have been isolated from various organisms, particularly from human, and their cDNA molecules have been cloned.

(0004)

A cDNA of human HS3OST has been disclosed in *J. Biol. Chem.*, 272, 28008-28019 (1997), and the cDNA described in the reference has been registered at GenBank as accession number AF019386.

(0005)

(Problems to be solved by the invention)

Although an enzyme which can transfer a sulfate group to a skeleton of heparin or heparan sulfate (hereinafter sometimes referred to as "heparin skeleton") is markedly useful because of its high possibility to be used in the enzymatic synthesis of heparin and heparan sulfate, such an enzyme has high substrate specificity so that it is necessary to carry out the synthesis efficiently by using various types of the enzyme for the purpose of industrially synthesizing various types of heparin and heparan sulfate. However, it cannot be said yet that there are sufficient variations of the enzyme capable of transferring a sulfate group to the heparin skeleton. That is, an object of the invention is to provide a novel sulfotransferase and also provide a means for obtaining the enzyme in a large amount by a convenient method through cloning of a cDNA encoding the amino acid sequence of the polypeptide to thereby increase variations of glycosaminoglycan which can be synthesized by enzyme chemistry and also contribute to the elucidation of structure-function relationship of glycosaminoglycan having heparin skeleton.

(0006)

(Means to solve the problems)

The present inventors have conducted intensive search on a DNA comprising a nucleotide sequence encoding glycosaminoglycan sulfotransferase capable of sulfating heparan sulfate and found a novel DNA having a nucleotide sequence encoding a polypeptide of the enzyme, and have confirmed that the glycosaminoglycan sulfotransferase can be obtained by expressing the DNA. Thus, the invention has been accomplished.

(0007)

That is, the present invention relates to the followings:

(Scope of the claims)

(1) A polypeptide which comprises the amino acid sequence represented by SEQ ID NO:2, or a polypeptide of a sulfotransferase which comprises an amino acid sequence having substitution, deletion, insertion or transposition of at least one amino acid in the amino acid sequence and has activity of transferring a sulfate group from a sulfate group donor to a glycosaminoglycan which is a sulfate group acceptor.

(2) The polypeptide according to (1), which consists of the amino acid sequence represented by SEQ ID NO:2.

(3) The polypeptide according to (1), which consists of amino acid numbers 37 to 346 in the amino acid sequence represented by SEQ ID NO:2.

(4) The polypeptide according to any one of (1) to (3), wherein the glycosaminoglycan is heparin or heparan sulfate.

(5) A sulfotransferase which comprises the polypeptide according to any one of (1) to (4) and has activity of transferring a sulfate group from a sulfate group donor to a glycosaminoglycan which is a sulfate group acceptor.

(6) A nucleic acid which encodes the polypeptide according to any one of claims 1 to 4 or the sulfotransferase according to (5).

(7) A nucleic acid which consists of the nucleotide sequence represented by SEQ ID NO:1.

(8) A nucleic acid which hybridizes with the nucleic acid according to (6) or (7) or a nucleic acid consisting of a nucleotide sequence complementary to the nucleotide sequence under stringent conditions.

(9) An expression vector which comprises the nucleic acid according to any one of (6) to (8).

(10) A recombinant which comprises the expression vector according to (9).

(11) A recombinant which comprises a host cell which is a eucaryotic cell into which the expression vector according to (9) is introduced.

(12) A process for producing a polypeptide or a sulfotransferase, which comprises culturing the recombinant according to (10) or (11), and recovering the polypeptide according to any one of (1) to (4) or the sulfotransferase according to (5) from the obtained culture.

(0008)

(Embodiments for carrying out the invention)

The embodiments of the invention are described below.

(1) Enzyme of the invention

The enzyme of the invention is a sulfotransferase which comprises a polypeptide comprising an amino acid sequence consisting of at least amino acid numbers 37 to 346 in the amino acid sequence represented by SEQ ID NO:2 and also has activity of transferring a sulfate group from a sulfate group donor to a glycosaminoglycan which is a sulfate group acceptor.

(0009)

Examples of the polypeptide according to the enzyme of the invention include a polypeptide consisting of the amino acid sequence represented by SEQ ID NO:2 and a polypeptide consisting of an amino acid sequence consisting of amino acid numbers 37 to 346 in the amino acid sequence represented by SEQ ID NO:2. It is preferred that the polypeptides are derived from a mammal, particularly desirably from human. Among the polypeptides, a polypeptide consisting of an amino acid sequence consisting of amino acid numbers 37 to 346 which excludes the presumed transmembrane region from the amino acid sequence represented by SEQ ID NO:2 (a region consisting of amino acid numbers 1 to 36 in SEQ ID NO:2) is particularly preferred since it becomes a so-called solubilized form which facilitates preparation of the protein.

(0010)

In general, it is known that the enzyme activity is maintained when one or plural (generally from 2 to 34) constituting amino acids in an amino acid sequence of an enzyme protein are substituted, deleted, inserted, added and/or transpositioned, so that it can regarded as a variant of the same enzyme, and in the case where partial mutations such as substitution, deletion, insertion or transposition of one or plural (generally from 2 to 34) constituting amino acids are also generated in the amino acid sequence represented by SEQ ID NO:2 of the substance of the invention, this can be regarded as a substance which is substantially identical to the enzyme of the invention, so long as it keeps the sulfate group transferring activity which is described later (such a polypeptide having partial mutations in the polypeptide comprising the amino acid sequence represented by SEQ ID NO:2 is described as "a modified polypeptide" for the sake of convenience). It is preferred that the amino acid sequence of the modified polypeptide has a homology of 90% or more, preferably 95% or more, more preferably 97% or more,

with the amino acid sequence represented by SEQ ID NO:2. The homology of the amino acid sequence can be easily calculated by using conventionally known computer software such as FASTA, and the software can also be provided by internet.

(0010)

Also, in the above-described enzyme of the invention, a saccharide chain can be linked to the protein, so long as its amino acid sequence is the same as described above and it has the above-described enzyme activity. That is, an embodiment of glycoprotein is included in the enzyme of the invention as a matter of course.

(000)

The sulfate group donor used in the reaction of the enzyme of the invention is not particularly limited, so long as it is a substance capable of transferring the sulfate group to a sulfate group acceptor, but 3'-phosphoadenosine 5'-phosphosulfate (active sulfate: hereinafter referred also to as "PAPS") which is generally known to function as a sulfate group donor in the living body is preferred due to a high possibility that it is an original sulfate group donor for the enzyme because it is an enzyme which is originally acted by the enzyme of the invention in the living body.

(0013)

Examples of the glycosaminoglycan include hyaluronic acid, chondroitin, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, heparin and the like, glycosaminoglycan having the so-called heparin skeleton such as heparan sulfate or heparin is particularly preferred as the sulfate group acceptor for the enzyme of the invention, and heparan sulfate is most particularly preferred. Also, as is also apparent from the following Examples, the enzyme of the invention does not substantially have activity of transferring a sulfate group to shark cartilage-derived chondroitin sulfate, chondroitin obtained by desulfating bovine bronchus-derived chondroitin sulfate, swine

skin derived dermatan sulfate and desulfated dermatan sulfate prepared by removing sulfate group from cockscomb-derived dermatan sulfate.

(0014)

It is possible to easily confirm the sulfate group transferring activity of the enzyme of the invention, for example, by carrying out the enzyme reaction in a buffer at a temperature of 20 to 40°C by using PAPS labeled with a label such as a radioisotope (^{35}S, ^{3}H (tritium) or the like) or a fluorescent material (a radioisotope is preferred since it does not generate steric hindrance in the substrate) and also by using a glycosaminoglycan which is a sulfate group acceptor, and then examining whether or not the acceptor is labeled with the label, for example, by checking the reaction solution after the reaction through the combination of a separation means such as gel filtration or high performance liquid chromatography (hereinafter also referred to as "HPLC") with a label-detecting means (radioactivity detecting means such as a scintillation counter or autoradiography when a radioisotope is used as the label, or detection by a fluorescence detector when a fluorescent material is used as the label).

(0015)

Since the enzyme of the invention obtained in this manner has activity of specifically transferring the sulfate group to a glycosaminoglycan, particularly to heparin and heparan sulfate, it is possible to use this as a sugar chain-modifying agent. For example, in coexistence of a sulfate group donor and a sulfate group acceptor (sugar chain) in a buffer of pH 6.5 to 8.0, such a sugar chain-modifying agent can modify the sugar chain by transferring a sulfate group from the sulfate group donor to the sulfate group acceptor. Such modification of the sugar chain is preferably carried out in coexistence of a protein protecting agent (for example, protamine hydrochloride)

because the sugar chain can be sufficiently modified while keeping the stability of the enzyme of the invention.

(0016)

(2) Peptide of the invention

The enzyme of the invention is a polypeptide having activity of transferring a sulfate group from a sulfate group donor to a glycosaminoglycan which is a sulfate group acceptor.

(0017)

Examples of the polypeptide of the invention include a polypeptide consisting of the amino acid sequence represented by SEQ ID NO:2 and a polypeptide consisting of an amino acid sequence consisting of amino acid numbers 37 to 346 in the amino acid sequence represented by SEQ ID NO:2. In the case where partial mutations such as substitution, deletion, insertion or transposition of one or plural (generally from 2 to 34) constituting amino acids are also generated in the former or latter amino acid sequence, this can be regarded as a substance which is substantially identical to the peptide of the invention, so long as it keeps the sulfate group transferring activity. Preferably, it has a homology of 90% or more, preferably 95% or more, more preferably 97% or more. The homology of the amino acid sequence can be easily calculated by using conventionally known computer software such as FASTA, and the software can also be provided by internet.

(0018)

The sulfate group donor and glycosaminoglycan in the polypeptide of the invention are the same as those described in the above enzyme of the invention (heparan sulfate and heparin being preferable and heparan sulfate being most preferable), and the

activity of transferring a sulfate group can be measured by a measuring method similar to the above method described in the enzyme of the invention.

(0019)

The polypeptide of the invention is substantially the same as the enzyme of the invention to which no sugar chain is bound (that is, the polypeptide of the enzyme of the invention).

(0020)

Also, since the polypeptide of the invention has activity of specifically transferring a sulfate group to a glycosaminoglycan, particularly heparan sulfate and heparin, in the same manner as the enzyme of the invention, it can be used as a sugar chain modifying agent.

(0021)

(3) Nucleic acid of the invention, expression vector of the invention and recombinant of the invention

The nucleic acid of the invention is a nucleic acid which encodes the enzyme of the invention or polypeptide of the invention.

(0022)

The nucleic acid of the invention is not limited to deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), so long as it encodes the enzyme of the invention or polypeptide of the invention, and it may be either single-stranded or double-stranded. However, since the above-described enzyme of the invention and polypeptide of the invention are human-derived amino acid sequences, it is preferable that it is a DNA capable of encoding a polypeptide in many organisms including human.

(0023)

The term "nucleic acid encoding" as used herein means both of a nucleic acid consisting of a nucleotide sequence complementary to the nucleotide sequence of a template chain to be used as the template for mRNA synthesis and a nucleic acid consisting of a nucleotide sequence of the template chain, generally in the transcription in protein (polypeptide) synthesis.

(0024)

Examples of the nucleotide sequence of such a nucleic acid include the nucleotide sequence represented by SEQ ID NO:1, a nucleotide sequence consisting of nucleotide numbers 109 to 1041 (a nucleotide sequence corresponding to the coding region of the amino acid numbers 37 to 346) in the nucleotide sequence represented by SEQ ID NO:1, and nucleotide sequences complementary to these nucleotide sequences, and nucleic acids consisting of such nucleotide sequences are included in the nucleic acid of the invention.

(0025)

In addition, it is known that a single-stranded nucleic acid hybridizes with a nucleic acid comprising a nucleotide sequence complementary thereto under certain conditions, and the nucleic acid of the invention includes nucleotide chains consisting of the nucleotide sequence represented by SEQ ID NO:1, a nucleotide sequence consisting of nucleotide numbers 109 to 1041 in the nucleotide sequence represented by SEQ ID NO:1, and nucleotide sequences complementary to these nucleotide sequences under stringent conditions.

(0026)

Examples of the stringent conditions include conditions of 42°C in the presence of 50% formamide, 5 × SSPE (sodium chloride/sodium phosphate/EDTA (ethylenediaminetetraacetic acid) buffer), 5 × Denhardt's solution, 0.5% SDS (sodium

dodecyl sulfate) and 100 μg/ml of denatured salmon sperm DNA, and under conditions substantially identical thereto. That is, the stringent conditions are conditions employed in the general hybridization of genes and included in the term "under stringent conditions" as used herein, so long as they are conditions used in the screening and the like which use Northern blotting, Southern blotting or hybridization.

(0027)

The DNA which consists of the nucleotide sequence consisting of nucleotide numbers 109 to 1041 in the nucleotide sequence represented by SEQ ID NO:1, which is one of the preferred illustrative embodiments of the nucleic acid of the invention, can be prepared by the method described in the following Examples, or since the complete nucleotide sequence thereof has been found by the invention, it can also be prepared by carrying out a polymerase chain reaction (hereinafter also referred to as "PCR") in the usual way, for example, by using a human-derived cDNA library as the template and using a 5' primer (SEQ ID NO:3) and 3' primer (SEQ ID NO:4). In the same manner, a DNA consisting of the nucleotide sequence represented by SEQ ID NO:1 can also be prepared by carrying out PCR using a primer represented by SEQ ID NO:5 as the 5' primer and a primer represented by SEQ ID NO:4 as the 3' primer.

(0028)

The expression vector of the invention contains the above-described nucleic acid of the invention, which is generally constructed in such a manner that the enzyme of the invention or polypeptide of the invention can be expressed in a host cell from the nucleic acid of the invention which is a DNA.

(0029)

The basal vector to be used as the above-described expression vector of the invention can be optionally selected by those skilled in the art according to the host cell

used, and the expression vector of the invention can be constructed by ligating the above-described nucleic acid of the invention to the thus selected basal vector in the usual way.

(0030)

In addition, in order to facilitate its secretion, isolation, purification and analysis in preparing the enzyme of the invention or polypeptide of the invention by expressing the expression vector of the invention, the enzyme of the invention or polypeptide of the invention may be constructed in such a manner that it can be expressed as a fusion protein with a marker peptide. The marker peptide means any peptide, for example, selected from the group consisting of a signal peptide (a peptide consisting of 15 to 30 amino acid residues, which is present in the N-terminus of many proteins and functioning intracellularly for the selection of a protein: e.g., OmpA, OmpT, Dsb or the like), protein kinase A, protein A (a protein of about 42,000 in molecular weight, which is a constituting component of *Staphylococcus aureus* cell wall), glutathione S transferase, His tag (a sequence of 6 to 10 histidine residues), myc tag (a sequence of 13 amino acid residues, derived from cMyc protein), FLAG peptide (a marker for analysis consisting of a sequence of 8 amino acid residues), T7 tag (a sequence of the first 11 amino acid residues of gene 10 protein), S tag (a sequence of 15 amino acid residues, derived from pancreatic RNase A), HSV tag, pel B (a sequence of 22 amino acid residues of *Escherichia coli* outer membrane protein pel B), HA tag (a sequence of 10 amino acid residues, derived from hemagglutinin), Trx tag (thioredoxin sequence), CBP tag (a calmodulin binding peptide), CBD tag (a cellulose binding domain), CBR tag (a collagen binding domain), β-lac/blu (β-lactamase), β-gal (β-galactosidase), luc (luciferase), HP-Thio (His-patch thioredoxin), HSP (heat shock protein), Lnγ (laminin γ peptide), Fn (fibronectin partial peptide), GFP (green

fluorescent peptide), YFP (yellow fluorescent peptide), CFP (cyan fluorescent peptide), BFP (blue fluorescent peptide), DsRed, DsRed2 (red fluorescent peptide), MBP (maltose binding peptide), LacZ (lactose operator), IgG (immunoglobulin G), avidin and protein G, and any one of these marker peptides can be used. Among these, signal peptide, protein kinase A, protein A, glutathione S transferase, His tag, myc tag, FLAG tag, T7 tag, S tag, HSV tag, pelB and HA tag are particularly preferred since expression of the enzyme of the invention and polypeptide of the invention by genetic engineering techniques and their secretion, isolation, purification and analysis become more easy.

(0031)

As the above host cell, it is possible to use either a procaryotic cell (e.g., *Escherichia coli* or the like) or a eucaryotic cell (e.g., yeast, insect cell, mammalian cell or the like). Particularly, in the case where a procaryotic cell is used as the host cell, saccharide chain addition and the like do not occur when the nucleic acid of the invention is expressed, so that the peptide of the invention to which saccharide chains are not added can be obtained. However, since the enzyme of the invention or polypeptide of the invention is an enzyme or polypeptide generally expressed in eucaryote, a eucaryotic cell is preferred as the host cell, and its preferred examples include insect cells (they are superior in terms of the large scale synthesis of the enzyme of the invention or polypeptide of the invention) or mammalian cells (they are superior in terms that they are cells in which the enzyme of the invention is originally expressed).

(0032)

The recombinant of the invention is a cell in which the vector of the invention constructed using a suitable basal vector into such a host cell in the usual way.

(0033)

(4) Enzyme production method of the invention

The enzyme production method of the invention is a method for producing the polypeptide of the invention or enzyme of the invention, wherein the recombinant of the invention is allowed to grow, and the polypeptide of the invention or enzyme of the invention is recovered from the thus obtained grown material.

The method for culturing the recombinant of the invention can be carried out by those skilled in the art by optionally selecting a method suitable for the host cell used in the recombinant. Also, the term "culturing" is a general idea of not only growing the recombinant in outside of a living body, for example, by using a culturing apparatus or a culturing tool, but also propagating it in the living body by administering the host cell to the living body.

(0035)

The culture in the production method of the invention includes a medium used for culturing and the cultured recombinant itself, as well as excrements, secretions, body fluids, tissues and the like of the living body obtained when the recombinant is grown in the living body.

(0036)

As the recovery of the polypeptide of the invention from the culture, it is possible to separate the polypeptide of the invention, for example, by a separation means such as gel filtration or HPLC which is based on the difference in molecular weight and a separation means such as an affinity column in which a sulfate group donor (PAPS or the like) in the enzyme reaction of the polypeptide of the invention is made into a solid phase, or in the case where the polypeptide of the invention is expressed as a fusion protein with a marker peptide, by a means for specifically adsorbing the marker peptide. For example, in the case where FLAG peptide is used as the marker peptide, it is possible to obtain the polypeptide of the invention easily as

its fusion protein with the FLAG peptide by using an affinity column in which an anti-FLAG antibody is made into a solid phase.

(0037)

(Examples)

The invention is described below more illustratively based on Examples.

1. Search of gene data base and determination of nucleotide sequence of the nucleic acid of the invention

Using a conventionally known human-derived heparan sulfate 3-O-sulfotransferase (HS3OST) gene, analogous genes from a gene data base were searched. The sequence used was SEQ ID NO:AFO19836 of the HS3OST gene. In this case, Blast [Altschul *et al.*, *J. Mol. Biol.*, 215, 402-410 (1990)] was used in the search.

(0038)

As a result, an analogous sequence was found in a genomic sequence GeneBank Accession No. AL 355498, and a novel gene having homology with the HS3OST gene was identified. It was estimated by a gene analyzing program (GENSCAN: manufactured by Stanford University) that this novel gene is encoded by two exons.

(0039)

(1) Confirmation of coding region of the polypeptide of the invention

Using Human Kidney Marathon-Ready cDNA (manufactured by CLONTECH), PCR (35 cycles of 94°C for 5 seconds and 68°C for 4 minutes) was carried out with the attached AP1 primer (AP1 and AP2 adapters are attached to both ends of a cDNA fragment) and a primer set up to a sequence moiety around the 5'-terminus of the second exon (GP-226: SEQ ID NO:6). Subsequently, nested PCR (40 cycles of 94°C for 5 seconds and 68°C for 4 minutes) was carried out with the AP2

primer attached to the Marathon cDNA and a primer set up to the sequence moiety (GP-224: SEQ ID NO:7). The PCR product obtained as the result was subjected to an agarose gel electrophoresis, and a band of about 450 b was recovered by using Gel Extraction Kit (manufactured by QIAGEN). As a result of the analysis of the nucleotide sequence of the thus obtained DNA fragment by a conventional method, a sequence of the second exon was confirmed in succession to a sequence of the first exon (N-terminal 36 amino acids were encoded). The sequence was the same as that predicted by the gene analysis program. Accordingly, it was confirmed that the coding region for the polypeptide of the invention is the sequence shown in SEQ ID NO:1 in which the first exon and the second exon are bound to each other.

(0040)

(2) Cloning of second exon

Based on the above results, those which are encoded by the first exon are only the N-terminal 36 amino acids. Since the second exon encodes the majority of the polypeptide of the invention, it was considered that the principal part of the enzyme including the active region is contained in the second exon (the polypeptide of the invention encoded by the second exon is called SFT-1 for the sake of convenience). Accordingly, cloning of the second exon moiety was carried out by using a genomic DNA as the template.

(0041)

Using Human Genomic DNA (manufactured by CLONTECH) as the template, PCR (35 cycles of 94°C for 15 seconds, 50°C for 30 seconds and 68°C for 1 minute) of a region containing the second exon was carried out. The primers used were set to the genomic sequences of an upstream moiety of the second exon (SFTex2F: SEQ ID NO:8) and a downstream moiety of the termination codon (SFTex2R: SEQ ID

NO:9). The thus obtained fragment of about 1 kb was purified in the usual way, and its nucleotide sequence was analyzed to confirm that the sequence of the second exon was obtained.

(0042)

2. Introduction of SFT-1 gene into expression vector:

In order to prepare a gene expression system, the second exon DNA obtained above was firstly introduced into an expression vector pDONR201 of the Gateway system manufactured by Invitrogen, and Bacmid was further prepared by the Bac-to-Bac system manufactured by Invitrogen. The details are explained below.

(0043)

(1) Preparation of entry clone for the novel sulfotransferase

Using the PCR product obtained above by amplifying the second exon as the template, PCT (30 cycles of 94°C for 15 seconds and 68°C for 3 minutes) was again carried out to obtain a DNA fragment for used in the Gateway system. The primers used were a 5' primer (SFTgateF2: SEQ ID NO:10) and a 3' primer (SFTgateRstop: SEQ ID NO:11), prepared by adding a sequence for Gateway system to a sequence close to the 5'-terminus of the second exon and a sequence close to the termination codon. The DNA fragment was purified in the usual way and introduced into pDONR201 by a BP clonase reaction to prepare an entry clone. The reaction was carried out by incubating 1 μl of the DNA fragment of interest, 1 μl (150 ng) of pDONR201, 2 μl of a reaction buffer, 4 μl of Tris-ethylenediaminetetraacetic acid (EDTA) buffer (hereinafter sometimes referred to as "TE") and 2 μl of BP clonase mix, at 25°C for 1 hour. The reaction was terminated by adding 1 μl of proteinase K and keeping at 37°C for 10 minutes.

(0044)

Thereafter, 5 μl of the above-described reaction solution was mixed with 100 μl of competent cells (*Escherichia coli* DH5α) to carry out transformation by a heat shock method, and then the cells were spread on the LB medium containing kanamycin. A colony was picked up on the next day and cultured in 3 ml of LB medium containing kanamycin, and then plasmid was extracted and purified using QIAprep Spin Miniprep Kit (manufactured by QUIAGEN). Using a part of the thus obtained plasmid, its nucleotide sequence was analyzed by a conventional method to confirm that the DNA of interest has been introduced.

(0045)

(2) Preparation of expressing clone

The above-described entry clone has attL which is a recombination region when λ phage is cut out from *Escherichia coli*, on both sides of its insertion site, and the insertion site is transferred to a destination vector by mixing LR clonase (a mixture of recombinases Int, IHF and Xis) with the destination vector so that a expressing clone is prepared. The specific steps are as follows.

(0046)

Firstly, 1 μl of the entry clone, 0.5 μl (75 ng) of pFBIF, 2 μl of LR reaction buffer, 4.5 μl of TE and 2 μl of LR clonase mix were allowed to react at 25°C for 1 hour, and the reaction was terminated by adding 1 μl of proteinase K and incubating at 37°C for 10 minutes (pFBIF-SFT-1 is purified by this recombination reaction). The pFBIF is prepared by inserting Igκ signal sequence (SEQ ID NO:12) and FLAG peptide (SEQ ID NO:13) into pFastBac1, and in order to insert Gateway sequence by inserting a DNA fragment obtained by primers OT20 (SEQ ID NO:15) and OT21 (SEQ ID NO:16) using OT3 (SEQ ID NO:14) as the primer into the *Bam*HI and *Eco*RI sites in the same manner as described above, a conversion cassette was inserted using Gateway Vector

Conversion System (manufactured by Invitrogen). The Igκ signal sequence was inserted to convert the expressed protein into secretion type, and the FLAG tag to facilitate its formation.

(0047)

Thereafter, 5 μl of the above-described reaction solution was mixed with 50 μl of competent cells (*Escherichia coli* DH5α), followed by transformation by a heat shock method, and then the cells were spread on the LB medium containing ampicillin. A colony was picked up on the next day and cultured in 5 ml of LB medium containing ampicillin, and then the plasmid (pFBIF-SFT-1) was extracted and purified using QIAprep Spin Miniprep Kit (manufactured by QUIAGEN). Using a part of the thus obtained plasmid, its nucleotide sequence was analyzed by a conventional method to confirm that the DNA of interest has been introduced.

(0048)

(3) Preparation of Bacmid by Bac-to-Bac system

Next, the sequence of SFT-1 was inserted into Bacmid capable of multiplying in insect cells, by carrying out recombination between the above-described pFBIF-SFT-1 and pFastBac using the Bac-to-Bac system (manufactured by Invitrogen). This system is a system in which a gene of interest is incorporated into Bacmid by a recombinant protein produced from a helper plasmid, by simply introducing the gene of interest-inserted pFastBac into a Bacmid-containing *Escherichia coli* strain (*E. coli* DH10BAC) by using the recombination region of Tn7. In addition, since the lacZ gene is contained in Bacmid, it is possible to carry out the classical selection based on the color of colonies (blue (no insertion) - white (insertion)).

(0049)

That is, the above-described purified vector (pFBIF-SFT-1) was mixed with 50 μl of competent cells (*Escherichia coli* DH10BAC), followed by transformation by a heat shock method, the resulting cells were spread on the LB medium containing kanamycin, gentamicin, tetracycline, 5-bromoindolyl β-D-galactopyranoside (Bluo-gal) and isopropyl β-D-thiogalactopyranoside (IPTG), and then a white single colony isolated on the next day was further cultured to recover Bacmid.

(0050)

3. Introduction of Bacmid into insect cell and recovery of SFT-1

The above-described Bacmid obtained from a white colony was introduced into an insect cell Sf21 (manufactured by Invitrogen). That is, 9×10^5 cells/2 ml of the Sf21 cells were added to Sf-900IISFM (manufactured by Invitrogen) containing antibiotics in a 35 mm dish and cultured at 27°C for 1 hour to adhere the cells. As a solution A, 100 μl of Sf-900IISFM containing no antibiotics was added to 5 μl of Bacmid DNA. As a solution B, 100 μl of Sf-900IISFM containing no antibiotics was added to 6 μl of Cell FECTIN solution (manufactured by Invitrogen). Thereafter, the solution A and solution B were thoroughly mixed and incubated at room temperature for 15 to 45 minutes. After confirming that the cells were adhered, the culture medium was sucked and 2 ml of Sf-900IISFM containing no antibiotics was added. To a solution prepared by mixing the solution A and solution B (lipid-DNA complexes), 800 μl of Sf-900IISFM containing no antibiotics was added and thoroughly mixed. The culture medium was sucked from the cell suspension, and diluted lipid-DNA complexes solution was added to the cells and incubated at 27°C for 5 hours. Thereafter, the transfection mixture was removed, 2 ml of Sf-900IISFM containing antibiotics was added thereto, and then 72 hours thereafter, the cells were peeled off by pipetting to recover the cells and culture medium. This was centrifuged at 1,200 × g for 10

minutes, and the supernatant was stored in another tube (which was used as a first virus solution).

(0051)

Into a T75 culture flask, 6×10^6 Sf21 cells/15 ml Sf-900IISFM (manufactured by Invitrogen) (containing antibiotics) was put, 1 ml of the first virus solution was added thereto, followed by culturing at 27°C for 96 hours. After the culturing, the cells were peeled off by pipetting to recover the cells and culture medium. This was centrifuged at $1,200 \times g$ for 10 minutes, and the supernatant was stored in another tube (this was used as a second virus solution).

(0052)

Furthermore, the 6×10^6 Sf21 cells/15 ml Sf-900IISFM (manufactured by Invitrogen) (containing antibiotics) was put into a T75 culture flask, and 1 ml of the second virus solution was added thereto, followed by culturing at 27°C for 72 hours. After the culturing, the cells were peeled off by pipetting to recover the cells and culture medium. The mixture was centrifuged at $1,200 \times g$ for 10 minutes, and the supernatant was stored in another tube (which was used as a third virus solution).

(0053)

In addition, 100 ml of Sf21 cell suspension was put into a 100 ml capacity spinner flask at a density of 6×10^5 cells/ml, and 1 ml of the third virus solution was added thereto, followed by culturing at 27°C for about 96 hours. After the culturing, the cells and culture medium were recovered. The mixture was centrifuged at $1,200 \times g$ for 10 minutes, and the supernatant was recovered.

(0054)

Sodium azide, sodium chloride and calcium chloride were added to 10 ml of this culture supernatant to give final concentrations of 0.05% sodium azide, 150 mmol/l

sodium chloride and 2 mmol/l calcium chloride. After 50 μl of an anti-Flag antibody gel (Anti-Flag M1 monoclonal antibody Agarose Affinity Gel, manufactured by SIGMA) was added thereto, the mixture was gently stirred for 12 hours. After removing the supernatant by carrying out centrifugation (1,000 × g, 3 minutes, 4°C), washing was carried out three times with Tris buffered saline (TBS) containing 1 mmol/l calcium chloride. By removing excess washing solution by carrying out centrifugation (1,000 × g, 3 minutes, 4°C), an SFT-1-FLAG fusion protein was obtained and used as the enzyme agent of the invention for activity measuring use.

(0055)

4. Confirmation of SFT-1-FLAG and measurement of enzyme activity

(1) Confirmation of SFT-1

Using 5 μl of a gel to which the fusion protein (SFT-1-FLAG) purified above was bound, Western blotting was carried out in accordance with the usual method using a peroxidase-labeled anti-FLAG antibody (Anti-FLAG M2 Peroxidase, manufactured by SIGMA) (Fig. 1). As a result, it was confirmed that the fusion protein of FLAG protein with the novel sulfotransferase expressing in the culture supernatant was recovered and purified.

(0056)

(2) Measurement of the activity of the enzyme agent of the invention to transfer sulfate group to heparan sulfate and heparin

The fusion protein (SFT-1-FLAG) purified from a culture supernatant was added to 50 mmol/l of an imidazole-hydrochloric acid buffer (pH 6.8) containing 75 μg/ml of protamine hydrochloride, to which were subsequently added [^{35}S]-PAPS (5 × 10^5 cpm, manufactured by NEN) as the sulfate donor, and heparan sulfate (derived from bovine kidney: manufactured by Seikagaku Corporation) and heparan (derived from

swine intestines: manufactured by SIGMA) (500 μmol/l as the amount of hexosamine) as the sulfate acceptors, and the total volume was adjusted to 50 μl with distilled water. This reaction solution was allowed to react at 37°C for 20 minutes, and then the reaction was terminated by heating at 100°C for 3 minutes to deactivate the enzyme. After 130 μl of ethanol containing 1.3% potassium acetate and 0.5 mmol/l EDTA were added thereto, followed by stirring, and then the precipitate obtained by centrifugation was dissolved in 50 μl of distilled water. By again carrying out the ethanol precipitation and dissolution in 50 μl of water, filtration was carried out through a microfilter of 0.22 μm in pore size (manufactured by Millipore), followed by separation by HPLC. The separation was carried out by using G2500PW (manufactured by Tosoh) as the column, and 0.2 mol/l sodium chloride as the mobile phase, at a flow rate of 0.6 ml/min and at a column temperature of 35°C. The eluates from the column were recovered as fractions of every 0.3 ml, and the radioactivity of each fraction was counted by a scintillation counter (Fig. 2). As a result, peak of the radioactivity was detected at a position of the elution time of about 12 minutes. Since this elution time coincides with the elution time of heparan sulfate or heparin as the sulfate acceptor, it was confirmed that it shows the activity of transferring a sulfate group to these two acceptors.

(0057)

In addition, when the sulfate transferring activity was measured under the same conditions of the above-described activity measuring method using chondroitin sulfate D (shark cartilage origin: manufactured by Seikagaku Corporation), chondroitin (prepared by carrying out complete desulfation of bovine bronchus-derived chondroitin sulfate in accordance with the method described in *J. Am. Chem. Soc.*, 79, 152-153 (1957)), dermatan sulfate (swine skin origin: manufactured by Seikagaku Corporation) and desulfated dermatan sulfate (prepared by carrying out complete desulfation of

cockscomb-derived dermatan sulfate in accordance with the method described in *J. Am. Chem. Soc.*, 79, 152-153 (1957)) as the sulfate acceptors, the sulfotransferase activity was not observed upon these acceptors (Fig. 3). Accordingly, it was suggested that the SFT-1 has the specific activity for heparan sulfate and heparin.

(0058)

(Effect of the invention)

A nucleic acid comprising a nucleotide sequence encoding a polypeptide of a novel heparan sulfate sulfotransferase (SFT-1) capable of selectively transferring sulfate group to heparan sulfate is obtained by the invention. Furthermore, a polypeptide expressed by the nucleic acid is obtained.

Also, since a DNA comprising a nucleotide sequence encoding a polypeptide of STF-1 was obtained by the invention, it is expected that STF-1 can be mass-produced to an industrially applicable level.

(Sequence listing)

<110> NATIONAL INSTITUTE OF ADVANCED INDUSTRIAL SCIENCE AND TECHNOLOGY

<110> SEIKAGAKU CORPORATION

<120> Novel heparan sulfate sulfotransferase and nucleic acid encoding the same

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<160> 16

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20 25 30

agc ttg gat agg cta caa ccc att tgc ccc att gaa ggt cga ctg ggt 144
Ser Leu Asp Arg Leu Gln Pro Ile Cys Pro Ile Glu Gly Arg Leu Gly
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Val Arg Leu His Asp Leu Val Gln Gln Leu Pro Lys Ala Ile Ile Ile
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145 150 155 160

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Ile Thr Glu Glu Val Pro Glu Arg Ile Tyr Lys Met Asn Ser Ser Ile
165 170 175

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180 185 190

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195 200 205

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225 230 235 240

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Trp Leu Lys Tyr Phe Pro Ile Glu Gln Phe His Val Val Asp Gly Asp
245 250 255

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        35   40   45
Gly Ala Arg Thr Gln Ala Glu Phe Pro Leu Arg Ala Leu Gln Phe Lys
    50   55   60
Arg Gly Leu Leu His Glu Phe Arg Lys Gly Asn Ala Ser Lys Glu Gln
65   70   75   80
Val Arg Leu His Asp Leu Val Gln Gln Leu Pro Lys Ala Ile Ile Ile
                85   90   95
Gly Val Arg Lys Gly Gly Thr Arg Ala Leu Leu Glu Met Leu Asn Leu
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His Pro Ala Val Val Lys Ala Ser Gln Glu Ile His Phe Phe Asp Asn
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Phe Ser Tyr Pro Gln Gln Ile Thr Ile Glu Lys Ser Pro Ala Tyr Phe
145   150   155   160
Ile Thr Glu Glu Val Pro Glu Arg Ile Tyr Lys Met Asn Ser Ser Ile
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Lys Leu Leu Ile Ile Val Arg Glu Pro Thr Thr Arg Ala Ile Ser Asp
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Tyr Thr Gln Val Leu Glu Gly Lys Glu Arg Lys Asn Lys Thr Tyr Tyr
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Lys Phe Glu Lys Leu Ala Ile Asp Pro Asn Thr Cys Glu Val Asn Thr
    210   215   220
Lys Tyr Lys Ala Val Arg Thr Ser Ile Tyr Thr Lys His Leu Glu Arg
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Trp Leu Lys Tyr Phe Pro Ile Glu Gln Phe His Val Val Asp Gly Asp
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Arg Leu Ile Thr Glu Pro Leu Pro Glu Leu Gln Leu Val Glu Lys Phe
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Thr Arg Gly Phe Tyr Cys Leu Arg Phe Asn Ile Ile Phe Asn Lys Cys
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(0060)

(Brief description of the drawings)

(Fig. 1) It is a photograph showing Western blotting analysis of purified SFT-1-FLAG described in Examples.

(Fig. 2) It is a graph showing the activity of transferring a sulfate group to heparan sulfate and heparin. The circles show the sulfate group transferring activity to heparan sulfate, and the squares show the sulfate group transferring activity to heparin.

(Fig. 3) It is a graph showing the activity of transferring a sulfate group to chondroitin sulfate D, chondroitin, dermatan sulfate and dermatan. The open circles, the closed squares, the closed circles and the open squares show the sulfate group transferring activity to chondroitin sulfate D, chondroitin, dermatan sulfate and dermatan, respectively.

(Document name)
Drawings
(Reference No.)
J200200400

FIG. 1

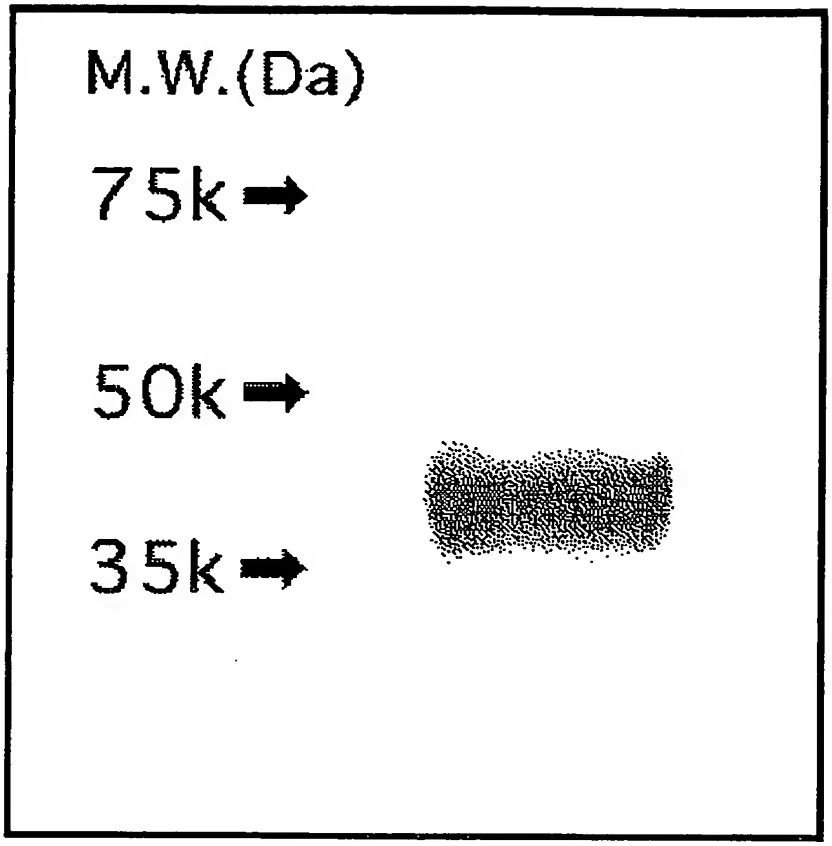
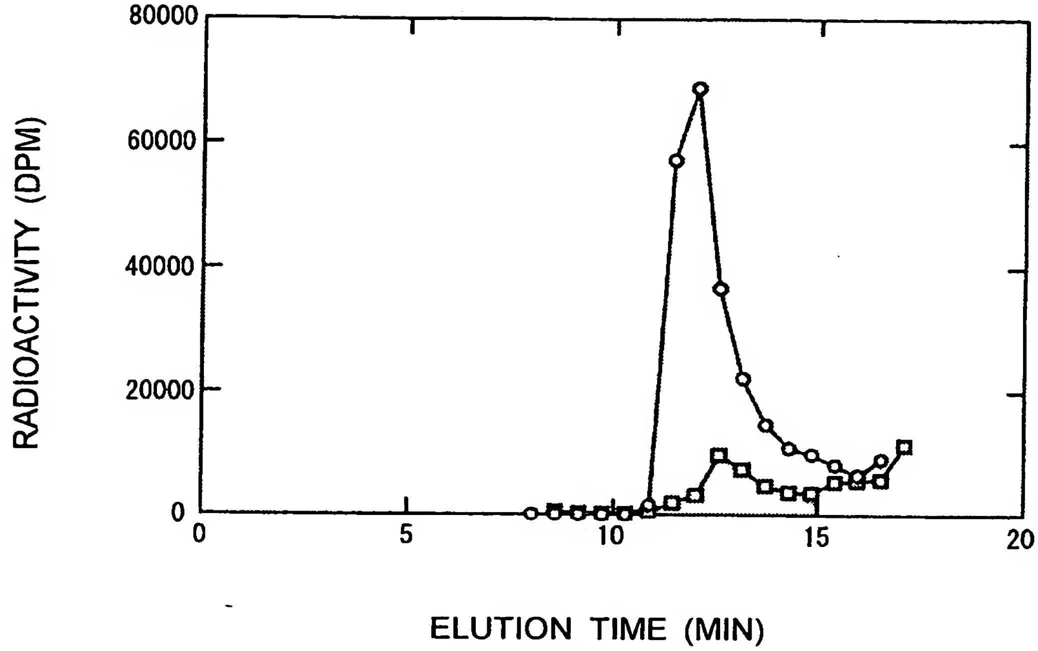


FIG. 2



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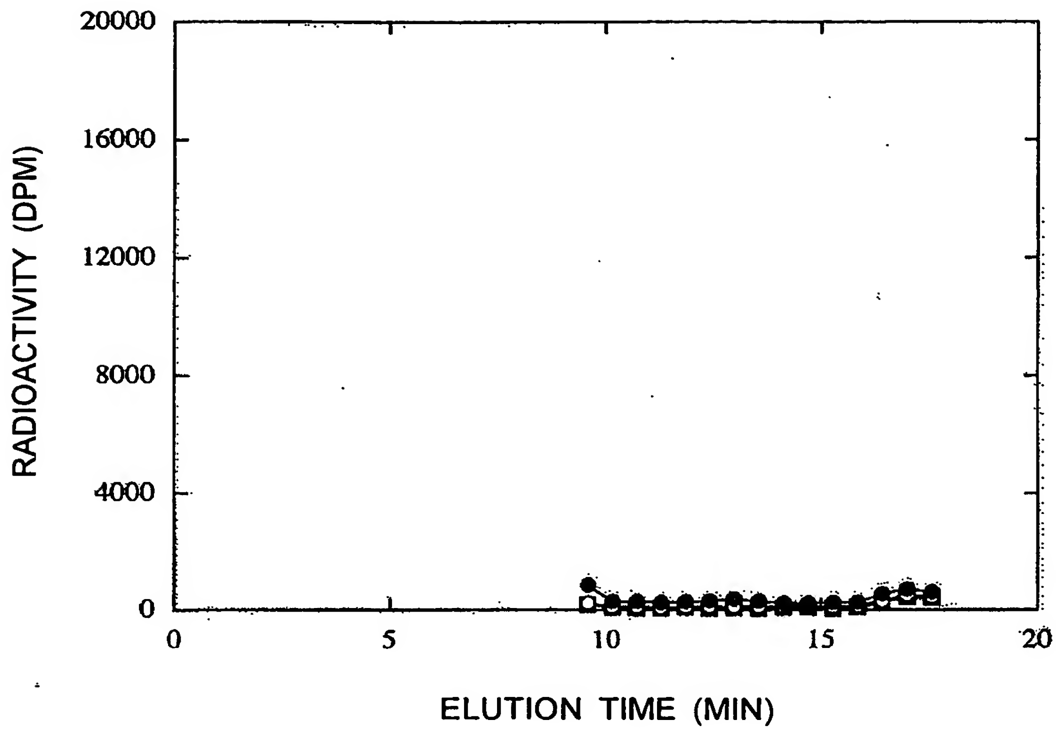
ELUTION TIME (MIN)

FIG. 3



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ELUTION TIME (MIN)

(Document name)

Abstract

(Reference No.)

J200200400

(Abstract)

(Problems)

They are to provide a novel sulfotransferase, a polypeptide thereof and a DNA encoding it.

(Means for solution)

A sulfotransferase which comprises a polypeptide comprising amino acid numbers 37 to 346 in the amino acid sequence represented by SEQ ID NO:2, and has activity of transferring a sulfate group from a sulfate group donor to a glycosaminoglycan which is a sulfate group acceptor, a polypeptide thereof, and a DNA encoding it.

(Selected drawing)

None